FACTORS INFLUENCING THE TOLERANCE OF PEANUT (*Arachis hypogaea* L.) PODS AND SEEDS TO COLONIZATION BY *Aspergillus flavus* LINK.

By

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Abstract of Dissertation Presented to the Graduate Council
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Factors Influencing the Tolerance of Peanut (*Arachis hypogaea* L.) Pods and Seeds to Colonization by *Aspergillus flavus* Link.

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A standard assay method was developed to determine if pods of different peanut breeding lines varied in susceptibility to *Aspergillus flavus* Link. For the assay, relatively constant moisture levels of 20-25% were required in both the shell and seed for a period of 14 days. Different initial moisture levels and incubation techniques were tested. The critical moisture required was obtained by adjusting pods to 25% and enclosing petri plates containing the rehydrated pods in plastic bags.

The pods were inoculated with different numbers of conidia of *A. flavus*. Similar percentages were infected whether the inoculation involved 10^5 conidia/ml or 5×10^7 conidia/ml. When seeds were inoculated, the percentage of colonization of seeds did not differ with inoculum levels between 10^6 and 10^7 conidia/ml. A suspension containing 5×10^6 conidia/ml was used for all further tests. The incubation period required for the determination of the number of colonized pods

was seven days. If invasion of the seed within the pod was to be examined, an additional seven days were required. All three isolates of A. flavus and one of A. oryzae that were used caused equal colonization of seeds and pods but differed in invasion of seeds within inoculated pods of four peanut lines. The A. flavus isolate No. 2999 was used for all further tests.

From the above tests a standard assay method was developed for studies of the tolerance of pods and seeds of peanuts to A. flavus. For the assay twenty grams of cured pods or 15 g of hand-shelled seeds were rehydrated to ca. 25% and 20% moisture, respectively, and were placed in 20 X 100 mm plastic petri dishes. The pods and seeds were inoculated with 1.5 ml and 0.5 ml, respectively, of a spore suspension (A. flavus, No. 2999) of 5 X 10^6 conidia/ml. The plates were swirled, placed in polyethylene bags and incubated at 25 C. After seven days the percentage of colonization of pods and seeds was determined. When invasion of seeds within inoculated pods was being tested, the plates were incubated for a total of 14 days.

The colonization of pods varied from 0-100% among the 17 breeding lines tested. More lines appeared to have highly tolerant pods than highly tolerant seeds. Individual pods that had been field cured were not uniformly colonized, but those that had been rapidly dried were uniformly colonized. In addition, in the later test all pods of all lines were colonized. If field cured pods were soaked in sodium hypochlorite, the percentage of pods colonized increased significantly as compared to those that were not soaked. When field cured pods were inoculated 10 hours after rehydration rather than within the usual 1 hour, colonization of pods was significantly lower. The tolerance in pods to colonization by *A. flavus* was associated with a low amount of aflatoxin.

The results of the above tests were consistant with tolerance of pods being caused by mycoflora of the shell. When triturated shells of different breeding lines were plated along with *A. flavus*, the predominent fungi from shells, *Penicillium* sp., *Rhizopus* sp. and *A. niger* suppressed the growth of *A. flavus*. Definite zones of inhibition of the growth of *A. flavus* were observed. When blocks of triturated shells suspended in rose bengal streptomycin agar (RBSA) were placed on plates of RBSA mixed with conidia of *A. flavus*, different fungi were observed growing from the blocks. These observations suggest that the antagonism of *A. flavus* in the shell was due at least partly to fungi present on or in the shell.

INTRODUCTION

A metabolite of the fungus Aspergillus flavus Link found in moldy peanut meal was responsible for the death of 100,000 turkeys in England in 1960 (8, 51). Since then this toxin, called aflatoxin, has been found in a wide variety of agricultural commodities grown in all parts of the world (24, 26).

Aflatoxin actually consists of mixture of four different chemical compounds of varying toxicity that have been designated as B_1, B_2, G_1 and G_2 (1). All four compounds are highly carcinogenic and sub-lethal doses have been associated with carcinomas of the liver in various test animals including ducklings, rats, and guinea pigs (12, 13, 37).

Aflatoxin has been reportedly produced by fungi other than those in the A. flavus group (24). However, only A. flavus and A. parasiticus Speare actually produce aflatoxins (60). Aspergillus flavus has been reported to contaminate and produce aflatoxin in many agricultural products including peanuts (24, 26).

Peanuts can be infected by A. flavus while the pods are developing in the soil (58, 59) or while the plants are curing in the field or when the pods or seeds are in storage (45). Most infections appear to occur during the windrow-curing process in the field especially when adverse weather delays harvest and subsequent artificial drying (4, 5, 42, 45).

Several production methods including use of digger-inverters, faster drying, better storage, and separation of damaged peanut pods from sound pods, have successfully reduced the seed contamination by *A. flavus* (35). However, many tons of farmer's stock peanuts are found each year that are visibly contaminated with *A. flavus* and thus must be diverted from food channels.

Another method that has been suggested for reducing *A. flavus* contamination is varieties that are tolerant to the fungus (40, 47). Tolerant lines are those whose seeds are not readily colonized by the fungus even though optimum conditions for invasion exist.

The objectives of this study were to investigate the tolerance to *A. flavus* reported from assays of hand-shelled seed to see if the so called tolerant peanuts were likely to be invaded before shelling and to determine if the shell might provide a barrier against the invasion of intact peanut pods by the fungus.

This study has been divided in to two parts. The first covers the development of an assay system to evaluate the tolerance of pods to *A. flavus* based on colonization. The second part of this study involves evaluation of different peanut breeding lines for the tolerance of pods to *A. flavus* based on the visible colonies of the test fungus on the surface of the pod, penetration through the shell, and invasion and/or colonization of the seeds within the intact pod. The nature of tolerance of pods of certain breeding lines to *A. flavus* has been investigated.

REVIEW OF LITERATURE

Aspergillus flavus Link has been reported to colonize and produce aflatoxin in many agricultural products including peanuts (24, 26). The growth of the fungus and the amount of aflatoxin produced varied not only with the substrates used but also with different environmental conditions (22, 26). After being heat-killed (85 C for 12 hours), peanut seeds were inoculated with conidia of *A. flavus* and were held under different relative humidity levels (22). The fungus grew well and produced maximum alfatoxin at 97-99% relative humidity. The limiting relative humidity for aflatoxin production was 85%. The moisture content of seeds also has been reported to be critical for successful colonization by *A. flavus* (13, 14). Seed moisture of 20-30% was reported to be optimum for maximum colonization of seed by *A. flavus* (3). However, when pods were inoculated, maximum colonization of the seed within was reported to occur at a narrower moisture range of 20-25%. A seed moisture of 9% was found to limit aflatoxin production in seeds inoculated with *A. flavus* (4).

Although *A. flavus* produces aflatoxin under a relatively narrow range of seed moisture contents and atmospheric relative humidities, it grows well and produces aflatoxin in a wide range of temperatures, 14-43 C and 14-40 C, respectively. The optimum temperature for production of aflatoxin varies from 24-35 C, depending on the other environmental conditions (15, 16, 17, 21, 22, 23).

Atmospheric gases may also influence production of aflatoxin in peanuts. Increased concentrations of CO_2 decreased production of aflatoxin (25, 50). Unfortunately, the flavor quality of peanut seeds is affected adversely by high concentrations of CO_2.

Certain fungi have been reported to inhibit growth of *A. flavus* (58, 59) and production of aflatoxin (24). Wells *et al.* (59) reported that under gnotobiotic conditions the colonization of the peanut pericarp by *A. flavus* was reduced in the presence of *Trichoderma viridae* Person ex. Friez and stimulated in the presence of *Penicillium funiculosum* Thom. However, when both *T. viridae* and *P. funiculosum* were present, the antagonistic effect of the former on *A. flavus* was nullified by the latter. Prior inoculation of peanut substrate with *A. niger* Van Tiegham was reported to reduce production of aflatoxin by 12% following subsequent inoculations with *A. flavus* (10). Conversely, Jackson (49) reported a reduction of naturally occurring fungi in seeds when *A. flavus* was inoculated to pods that had been contaminated naturally with *Rhizopus stolonifer*, *A. niger*, *Sclerotium bataticola* and *Penicillium citrinum*.

The condition of the pod affects its susceptibility to *A. flavus* (2). Pods that were overmature or brittle at the time of harvest were associated frequently with contamination by *A. flavus* (5). Overmature seeds were reported to be more susceptible than mature seeds to colonization by *A. flavus* following inoculation (47). This increased susceptibility has been attributed to a loss in physiological activity of the seed (5, 44). Diener and Davis (22) reported increased colonization and production of aflatoxin in heat-killed seeds as compared to seeds that had not been so treated.

The penetration through different layers of the peanut seed by *A. flavus* has been studied. Peanut seeds were invaded directly after inoculation with *A. flavus* (41). The fungus was isolated from cotyledons within 24 hours following inoculation with *A. flavus* (47). It has been isolated from the inner surface of the shells seven days after inoculation of intact pods and was found in the seed one week later (14). When pods of peanut plants growing under gnotobiotic conditions were inoculated with *A. flavus*, the fungus penetrated the shells but was limited in its invasion of the seeds to the testa (59). In a similar gnotobiotic test, Wells and Kruetzer (58) reported that *A. flavus* could invade flowers and remain associated with the developing pod tissue.

Several possibilities for reducing aflatoxin or for preventing *A. flavus* from attacking peanut seeds have been explored (35). Since most of the contamination has occurred when the pods are curing in the field (10), attempts have been made to develop more efficient curing methods (35, 43). Such techniques have reduced but not eliminated the incidence of aflatoxin (11). Fungicides have been evaluated under laboratory conditions (28). However, commercial use of fungicides has not been feasible due to lack of efficiency (49).

Various methods have been used to detect the presence of *A. flavus* or aflatoxin in the pods and seeds of farmer's stock peanuts in order to identify those lots containing aflatoxins and to divert them from food channels (35, 48). The pods and/or seeds containing visible growth of *A. flavus* are culled segregation III and must not be used for edible purposes (18).

Attempts are being made to breed or select varieties that are resistant or tolerant to seed colonization by *A. flavus* (40, 46).

Mixon and Rogers (46) reported that two peanut breeding lines out of hundreds tested appeared to be highly tolerant. Less than 5% of the tolerant seeds had colonies following inoculation with *A. flavus*.

LaPrade *et al.* (40) reported four peanut breeding lines that were less than 15% colonized in similar assays. Seeds of a peanut breeding line, Asiriya Mwitunde, that were reported to resist the production of aflatoxin (36, 56) were found later to contain substantial amounts of aflatoxin following *A. flavus* inoculations (27). McDonald and Harkness (43) assayed four breeding lines for resistance in the seeds to the production of aflatoxin by *A. flavus* and found all varieties to be equally susceptible.

The nature of tolerance of peanut seeds to invasion by *A. flavus* has been attributed mainly to structural factors. LaPrade and Bartz (39) reported that tolerant peanut seed possessed thicker seed coats and heavier cuticular wax than did more susceptible seed. Taber *et al.* (57) reported larger hila and thicker palisade like layers in the seed coats of tolerant seeds. Dieckert *et al.* (19) reported that the testae of tolerant seeds served as structural barriers to invasion by the fungus. Benedict *et al.* (7) reported greater rates of water uptake through hilum by susceptible seeds.

PART I

ASSAY METHOD

INTRODUCTION

The general method used to screen peanut breeding lines for tolerance to *Aspergillus flavus* Link involves rehydration of hand-shelled seeds, inoculation with conidia of the fungus and incubation for 7-14 days at 25 C (40, 46). The level of tolerance exhibited by a particular line is based upon the percentage of seeds with visible colonies of *A. flavus* at the end of the incubation period. This technique was adopted for use with intact pods where the tolerance of the pod was based upon visible colonization of the pod.

Colonization of peanut pods or seeds by *A. flavus* is influenced by many environmental factors. Moisture content seems to be the most important (22, 47). Although the minimum and maximum moisture contents that will support the growth of *A. flavus* are interrelated with temperature, the optimum range for colonization of peanut seed at ca. 25 C was reported to be 20-25% (14, 47). The percentage of seeds colonized was affected more by a reduction below 20% moisture than by a similar percentage increase above 25% (14, 22). Thus, in an assay of the tolerance of pods to *A. flavus* all portions of the pod (shell and seed) must be above a 20% moisture content. Furthermore, this moisture content should be maintained until the fungus has had an opportunity to invade the cotyledons of the seed.

Another factor in the percentage of peanut pods colonized by *A. flavus* is the amount of inoculum present. The inoculum concentrations used in the assays for tolerance of seed to *A. flavus* has been standardized (14, 38, 46), however, the influence of different spore concentrations on the percentage of seeds and pods colonized by *A. flavus* has not been reported.

The purpose of this study was to develop a standard assay method to test the pods of different peanut breeding lines for their tolerance to *A. flavus*.

In this investigation the influence of a few factors such as different spore concentration, different incubation period, and different isolates of *A. flavus* on percentage colonization of pods and seeds have been evaluated. To maintain a seed moisture of 20-25% during incubation different initial pod moistures and different incubation techniques have been evaluated.

MATERIALS AND METHODS

Peanut plants were grown with standard production techniques in 6' X 20' plots. At or near maturity the plants were dug with a Goodrich 2-row-digger-shaker-inverter and within 3 hours were placed on stack-poles and covered with tarpolins. After 4-6 weeks the cured pods were picked with a Ce-Co-Co plot picker and cleaned in a conventional peanut pod cleaner. The cleaned pods were stored in paper bags in the laboratory until used.

For moisture determinations shells and seeds were seperately dried in a forced air oven at 90 C for 72 hours. The pods were shelled just before drying.

Pods or seeds were adjusted to designated moisture levels (rehydration) by a modification of a method for seeds described by Mixon and Rogers (46). Seeds or pods were immersed in distilled water for ca. 25 minutes, the water was decanted, and the pods or seeds were placed in tared plastic petri dishes (20 X 100 mm). Additional distilled water was added to provide the calculated final weight for the moisture level desired. The plates were then either enclosed in polyethylene bags (6-12 plates/bag) or directly placed into an incubator set at 25 C. Initial moisture levels of 6% and 5% were assumed for all peanut pod and seed samples, respectively. However, the measured moisture level of the cured pods varied from 5.6-7% among the different breeding lines tested.

The isolates of A. flavus (No. 2999, 3694, 5519) and one isolate of A. oryzae (No. 482) obtained from the National Regional Research Laboratory (NRRL) at Peoria, Illinois, were used for inocula in the colonization tests. Isolates No. 2999 and No. 3694 are reported to produce aflatoxin in peanuts (54), but No. 5519 (Dr. R. J. Bothast, Personal Communication) and No. 482 (9) do not produce aflatoxin in culture media or in corn.

Inocula were prepared from 10-15 day old cultures grown on Czapex dox agar in 20 X 100 mm plastic petri dishes. The conidia of isolates No. 3694, 5519 and No. 482 were harvested by flooding the cultures with a 0.02% aqueous solution of Tween 20, while those of isolate No. 2999 were removed by scraping the surface of the cultures gently with a sterile inoculation needle. Spore concentrations were measured with a hemacytometer.

Pods or seeds were inoculated by pipetting 1.5 and 0.5 ml of the standardized suspensions, respectively, into the plates. A spore suspension of 5 X 10^6 conidia/ml was used throughout the study, unless otherwise indicated. The plates were swirled thoroughly, enclosed in polyethylene bags and incubated at 25 C for indicated lengths of time.

To determine the percentages of seed or pod colonization, the number of seeds or pods with visible colonies of the test fungus was noted after seven days of incubation. To determine the percentage of invasion of pods and seeds within intact pods, the inoculated pods (disinfested with 0.5% NaOCl for 5 minutes before inoculation) that had been incubated for up to 14 days were soaked in 1.5% sodium hypochlorite (NaOCl) for 5 minutes, rinsed with distilled water and either plated on malt salt agar (MSA) or shelled. The seeds from the shelled pods were soaked in 1.5% NaOCl again, washed twice with distilled water, and then plated on malt salt agar. After the pods or seeds were incubated at 25 C for five days, invasions were determined by noting the number of pods or seeds with visible colonies of *A. flavus*.

Shell penetration was determined by isolating the test fungus from the inner surface of the shells of inoculated pods. A 2-3 mm block of rose bengal streptomycin agar (RBSA) media (6) modified by excluding the botran was touched to the inside surface of the shell to trap the fungus. The blocks were then plated (5 blocks/plate) on RBSA media and incubated at 25 C. After five days the number of blocks with colonies of the test fungus was observed.

RESULTS

Critical pod and seed moisture levels. The standard assay for tolerance of peanut seed to *A. flavus* involves incubating rehydrated (to 20% moisture) peanut seed in petri dishes in a standard incubator. When pods were adjusted to 20% moisture and so incubated, the moisture level of the seed did not reach 20% but rather reached about 18% after three days of incubation (Table 1). The moisture level of the seeds dropped to 10% after 14 days of incubation. The shells contained more moisture than the seeds at all observations and at equilibrium (1-7 days) had 3 to 5% more moisture.

Since the moisture level of the seeds within the pods did not reach the 20% moisture level, a comparison of various levels of initial pod moisture to seed moisture following a 14-day incubation was made. Even though the pods were adjusted to 30% moisture, the level of moisture in the seed was not at 20% following a 14 day incubation (Table 2). The rapid loss of moisture during incubation was observed in all four initial moisture adjustments. As an additional treatment in the preceeding test, pods adjusted to 25% moisture were enclosed in polyethylene bags. The pod as well as the seed moisture were above 20% following 14 days of incubation when pods were enclosed in bags (Table 3).

Influence of spore concentration on seed and pod colonization. Seeds of three peanut breeding lines (BRL) were inoculated with different concentrations of conidia of *A. flavus* and observed for colonization of seeds.

The percentage colonization of seeds varied significantly among spore concentrations between 10^5 and 10^7 conidia/ml (Table 4). The average colonization of seeds at 10^7 conidia/ml was significantly higher than that at 10^5 conidia/ml but colonization at neither level differed from that at 10^6. When individual breeding lines were analyzed, the colonization of seeds in BRL No. 19 was significantly higher at 10^7 conidia/ml than at 10^6 or 10^5 conidia/ml.

Pods of four peanut breeding lines were inoculated with different concentrations of conidia of *A. flavus* and observed for colonization. The percentage colonization of pods varied significantly among the different concentrations tested (Table 5). The mean percentages of colonization of pods of all the breeding lines were significantly lower at 10^4 conidia/ml than were those from the higher concentrations. The colonization of pods was highest at 10^6 conidia/ml, and was less at 10^4 or 5 X 10^7 conidia/ml in BRL No. 15. In BRL No. 26 the percentage of colonization of pods was lower at 10^4 conidia/ml than that at 10^5 and 10^6 conidia/ml.

Influence of incubation period on the invasion of pods, shells, and seeds by A. flavus. The pods of BRL No. 14 inoculated with *A. flavus* were incubated for different lengths of time and observed for the penetration of pods, shells, and seeds. The optimum incubation time for maximum invasion of pod, shell, and seed were 7, 4, and 14 days, respectively (Table 6). The percentage of different parts of the pod that were invaded seemed to decrease at incubation continued after the respective optimum time. The pods, shells, and seeds were initially invaded in 2, 2, and 14 days, respectively. However, a small percentage of the shells appeared invaded in one day. Colonies of *A. flavus* were visible on the pods four days after incubation and at that time the percentage of pods colonized and the percentage pods invaded were identical to each other.

Influence of different isolates of A. flavus and A. oryzae on colonization of seeds and pods and invasion of seeds. The seeds and pods of four breeding lines were inoculated with different isolates of *A. flavus* and *A. oryzae* and observed for variation in the percentage of colonization of seeds and pods, and the percentage of invasion of seeds. The various isolates tested here differed in their ability to invade seeds (Table 7) but not in their ability to colonize pods and seeds (Table 8). The isolate No. 2999 invaded more seeds than did the other isolates. However, when the different isolates were compared within each breeding line, the isolate of *A. oryzae* differed significantly from the isolates of *A. flavus* in the colonization of pods of BRL No. 15. The isolate No. 2999 differed from two other isolates of *A. flavus* but was similar to the isolate of *A. oryzae* in invasion of seeds of BRL No. 19.

Table 1: The percentage moisture content of pods, shells, and seeds of intact pods of two peanut breeding lines adjusted to 20% moisture, placed in plastic petri plates and incubated for different periods at 25 C.

Incubation period - days[y]	Percentage of moisture content[x]					
	BRL No. 74-7[z]			BRL No. 74-16		
	Pod	Shell	Seed	Pod	Shell	Seed
0 ck	5.8	9.5	5.0	5.6	9.4	4.8
0	19.7	51.7	7.6	19.3	50.8	8.3
0.5	19.5	48.4	9.3	19.6	47.3	9.8
1	18.9	22.4	18.1	19.6	30.0	17.4
3	17.3	19.7	16.8	18.7	22.0	18.0
7	16.6	17.4	15.0	15.6	18.1	15.1
14	10.7	15.6	9.7	11.3	16.6	10.3

[x] Each value is the average of three 20 g pod samples.

[y] Identification of peanut breeding lines (BRL) included in appendix.

[z] 0 ck = moisture percentages before the pods were rehydrated.
0 = moisture percentage immediately after rehydration.

Table 2: The percentage moisture content of pods and seeds of intact pods of two peanut breeding lines adjusted to four different moisture levels, placed in plastic petri plates, and incubated for 14 days at 25 C.

Per cent initial moisture	Percentage moisture content[x]			
	BRL No. 74-7		BRL No. 74-16	
	Pod	Seed	Pod	Seed
15	8.3	7.5	8.5	7.6
20	10.7	9.7	11.3	10.3
25	16.4	15.8	16.3	15.6
30	18.5	18.2	18.6	18.1

[x] Each value is the average of three 20 g pod samples.

Table 3: The percentage moisture content of pods, shells, and seeds of intact pods of two peanut breeding lines adjusted to 25% moisture, placed in plastic petri plates, enclosed in polyethylene bags and incubated for 7 or 14 days at 25 C.

Breeding line No.	Incubation period (days)	Percentage moisture content[x]					
		Enclosed[y]			Not enclosed		
		Pod	Shell	Seed	Pod	Shell	Seed
74- 7	7	24.2	24.6	22.2	20.8	21.3	20.7
	14	23.9	24.4	22.1	16.4	18.8	15.8
74-16	7	24.2	24.7	24.2	20.3	21.4	20.1
	14	24.2	24.5	22.7	16.3	19.3	15.6

[x] Each value is the average of three 20 g pod samples.

[y] Enclosed = the plates that were enclosed in polyethylene bags (6-12 plates/bag) during incubation.
Not enclosed = the plates that were not enclosed in polyethylene bags.

Table 4: The percentage colonization of seeds of three peanut breeding lines by *Aspergillus flavus* (isolate No. 2999), following inoculations with three different concentrations of conidia.

Spore conc. conidia/ml	Percentage colonization of seeds			
	Breeding line No.			
	75-7	75-15	75-19	Mean
10^5	6a[x]	7a	25 b	13 b
10^6	21a	15a	21 b	19ab
10^7	27a	28a	35a	30a

[x] Values (average of three 15 g seed samples) within columns followed by different letters are significantly different (*P*=0.05 by Duncan's New Multiple Range Test).

Table 5: The percentage colonization of pods of four peanut breeding lines by *Aspergillus flavus* (isolate No. 2999), following inoculations with five different concentrations of conidia.

Spore conc. conidia/ml	Percentage colonization of pods Breeding line No. 74-7	74-15	74-16	74-26	Mean
10^4	0a[x]	41 c	43a	0 b	20 b
10^5	3a	75ab	85a	11a	39a
10^6	10a	87a	76a	11a	42a
10^7	3a	81ab	90a	5 b	38a
5×10^7	13a	67 bc	58a	14a	37a

[x] Values (average of three 20 g pod samples) within columns followed by the different letters are significantly different (*P*=0.05, by Duncan's New Multiple Range Test). Data were transformed to arcsin values for analysis.

Table 6: The percentage of invasion of pods, shells, and seeds by *Aspergillus flavus* (isolate No. 2999), following inoculation of intact pods of peanut breeding line 75-14 and incubation for different periods of time.

Incubation period (days)	Percentage invasion[x]		
	Pod	Shell	Seed
0.5	0	0	0
1	0	3	0
2	53	30	0
4	80	65	8
7	85	51	17
14	69	37	29
21	66	15	22

[x] Each value is the average of three 20 g pod samples.

Table 7: The percentage colonization of seeds of four different peanut breeding lines by the test fungus, following inoculation with three different isolates of *Aspergillus flavus* or one of *A. oryzae*[x].

	Percentage colonization of seeds[y]			
	Breeding line No.			
Isolates	75-7	75-15	75-16	75-19
A. flavus				
2999	21	22	21	15
3794	17	17	22	24
5519	22	17	19	22
A. oryzae				
482	27	22	21	25

[x] Analysis of variance indicated no significant difference among isolates or among breeding lines (at $P=0.05$).

[y] Each value is the average of three 15 g seeds.

Table 8: The percentage colonization of pods, and the percentage invasion of seeds, by the test fungus, following inoculation of intact pods of four different peanut breeding lines with three different isolates of *Aspergillus flavus* or one of *A. oryzae*.[x]

	Breeding line No.				
Isolates	75-7	75-15	75-16	75-19	Mean
			Per cent pod colonization		
A. flavus					
2999	100a[y]	56a	100a	2a	
3794	100a	59a	100a	2a	
5519	100a	49a	100a	0a	
A. oryzae					
482	100a	16 b	97a	0a	
		Per cent seed invasion of inoculated pods			
A. flavus					
2999	40a[y]	21a	29a	9a	29a
3794	7a	9a	20a	1 b	16 b
5519	23a	14a	17a	1 b	19 b
A. oryzae					
482	10a	8a	23a	5ab	18 b

[x] Analysis of variance indicated significant ($P=0.05$) difference among isolates only in pod but not in seed colonization. Data were transformed to arcsin values for analysis.

[y] Values (average of three 20 g pod samples) within columns followed by the different letters are significantly different ($P=0.05$, by Duncan's New Multiple Range Test).

DISCUSSION

A technique designed for comparing the susceptibility or tolerance of different plants to a fungus should ensure ideal environmental conditions for infection by the fungus. In the assay of pods and seeds of different peanut lines for tolerance to *A. flavus*, the moisture content of the pods or seeds is the critical factor of the environment that needs to be controlled. The optimum range for colonization of peanut pods and seeds by *A. flavus* is 20-25% moisture (14, 46). In one assay that was described for determining the tolerance of seeds to *A. flavus*, the seeds were rehydrated to 20% moisture by soaking the seeds in water and then incubated in an environmental cabinet set at 100% relative humidity (46). When pods in petri plates adjusted to 20% moisture were incubated in a standard incubator (no control of relative humidity), the moisture levels of pods and seeds never reached 20% and was less than 12% by 14 days. The moisture content of the shell was higher than that of the seed as expected (34). Even though the pods were adjusted to 30% moisture in a succeeding test, the seed moisture was still below 20% by 14 days. If, however, the petri plates containing pods adjusted to 25% moisture were enclosed in polyethylene bags, the seed moisture was within the optimum range of 20-25% at 14 days. Thus, plates containing pods or seeds rehydrated to 25% moisture were enclosed in polyethylene bags in the assay method reported here. Other factors involved with the assay of peanut pods for tolerance to *A. flavus* that needed consideration were concentration of inoculum used and duration of the incubation period.

Colonization of both the inoculated seeds and the inoculated pods was influenced by the different inoculum concentrations used. Although the mean percentage of seeds colonized following inoculation of seeds with 10^5 conidia/ml differed from those inoculated with 10^7 conidia/ml, neither differed from those inoculated with 10^6 conidia/ml. Thus, it is evident that the spore concentration should be defined. The optimum inoculum level required for seed colonization could not be determined from the data available. An inoculum concentration of 5 X 10^6 conidia/ml, a concentration previously used by LaPrade (38) has been adopted here.

The colonization of pods was lower at 10^4 conidia/ml than at 10^5 conidia/ml. The reasons for the decrease in colonization of some lines at higher inoculum concentrations are unknown. There was no difference in colonization of pods when the inoculum concentration was between 10^5 conidia/ml and 5 X 10^7 conidia/ml. A spore suspension of 1.5 ml containing 5 X 10^6 conidia/ml was used to inoculate each of the 20 g samples of pods in further studies.

The colonies of _A. flavus_ appeared on inoculated pods after four days of incubation. However, the maximum colonization occurred in seven days. Hence, the incubation period of seven days has been used for further studies involving colonization of pods. An incubation period of seven days also has been used by other workers (38, 46) to study colonization of seeds. The optimum incubation time for maximum penetration of shells and seeds is four and 14 days, respectively. The reduction in colonization of pod and invasion of seeds noted during longer than optimum incubation periods may be due to rapid growth of fungi that were present (natural contaminants) in the shells and seeds.

One other factor concerning the assay that was studied involved the isolate of *A. flavus* used. This was done for two reasons, first to determine if the percentage of pods and seeds colonized was altered by use of different isolates; and secondly to determine if a non-toxin producing isolate could be used for screening peanut lines for tolerance to *A. flavus*. Seed and pod colonization did not differ among different isolates of *A. flavus* and *A. oryzae* tested here. However, when pods were inoculated with different isolates, the percentage of seeds invaded was higher following inoculation with isolate No. 2999 than with other isolates. If colonization of pods and seeds were being considered the non-toxin producer No. 5519 could be substituted for the toxin producing strain. Although *A. flavus* isolate No. 2999 was slightly more pathogenic and has been used by others to study tolerance (40, 46) the isolate No. 5519 would be much safer to use in a routine screening study and its use should be encouraged.

PART II

TOLERANCE OF PEANUT PODS AND SEEDS TO ASPERGILLUS FLAVUS LINK AND SOME OF THE FACTORS INFLUENCING THE TOLERANCE

INTRODUCTION

Peanut pods and seeds may be invaded by *A. flavus* at any one or more of various stages of pod development (58, 59) and/or after digging while the peanuts are curing in the windrow (3, 4, 30, 31, 45). Most invasion and subsequent contamination with aflatoxin is believed to occur when curing is delayed, especially after the seed moisture content has dropped below 30% (45). Several methods for reducing aflatoxin in seeds have been explored. At the present time, however, none of the methods are adequate (35). One solution that would avoid the problem of aflatoxin in peanut seed would be to grow peanuts that are resistant to *A. flavus*.

Peanut accessions have been screened for resistance to colonization by *A. flavus*. Mixon and Rogers (46) reported two peanut accessions with less than 5% colonization of seeds following rehydration, inoculation and incubation for seven days. Using similar assay methods LaPrade *et al*. (40) reported that four peanut breeding lines out of 165 tested had less than 15% colonization of seeds.

Most peanut seeds, however, are invaded while still in the shell (32). To successfully invade the seed, the fungus must first penetrate the shell. The possibility that the shell (pericarp) might help prevent the fungus from reaching the seed has not been studied. However, a substantial reduction in the colonization of pods by A. flavus has been reported in gnotobiotically produced peanut pods that were inoculated with other fungi in addition to inoculation with A. flavus (59).

The mature peanut pod is devoid of any outer protective layers like a cuticle or an epidermis. The shell consists of three layers, exocarp, mesocarp and endocarp (55). The exocarp consists of a few layers of epidermis and hypodermis which are exfoliated before the pod is mature. The mesocarp consists of 10-11 veins and a network of small veins composed of sclerenchyma. The endodermis consists of many layers of spongy parenchyma cells around the seed. When the pod is mature, the endodermis is reduced to a thin white layer on the inner shell surface. A characteristic brown splotching on the inner surface of the shell has been observed. This was reported to be due to tanins (52). Although higher levels of water soluble pockets of tanins were found in mechanical and conductive tissues of the shell during all stages of pod development, the tannins in the mature shell have been reported to be less water soluble and more complex. In addition, the shell tanin differ chemically from seed coat tanin.

The purpose of this study was to determine how the shell affected colonization of the seed when inoculum was applied to intact pods. The peanut lines used included some that were tolerant to A. flavus in the intact seed assay and some that were susceptible.

In the present investigation a few peanut breeding lines were tested for their pod and seed tolerance to A. flavus (isolate No. 2999). The nature of tolerance of pods was studied by altering the pod surface mycoflora by different methods: pod surface treatment with sodium hypochlorite, rapid drying of the pods immediately after digging, and incubation of pods prior to inoculation at high pod moisture. The antagonism of shell mycoflora to *A. flavus* also has been studied.

MATERIALS AND METHODS

Pods and seeds of different peanut breeding lines were grown, harvested and dried as detailed in part I with the exception of the oven dried pods where the peanuts were hand harvested, washed, and dried in a forced air drier at 40±2 C for 12 or 48 hours. After drying, pod and seed moistures were determined as in part I.

Prior to inoculation pods and seeds were adjusted to 25% and 20% moisture contents, respectively, as detailed in part I. The pods that had been oven dried for 48 hours were rehydrated in a similar manner while those that had been dried only for 12 hours did not need to be rehydrated.

The pods and seeds were inoculated by pipetting, 1.5 and 0.5 ml respectively, of a 5×10^6 conidia/ml suspension of *A. flavus* isolate No. 2999 into the plates. The suspension was prepared from 10-15-day old Czapex's agar cultures of the fungus. Conidia on the surface of the culture had been scraped gently with an inoculation needle into a beaker containing .02% aquous solution of Tween 20. The resulting suspension was adjusted to 0.8 O.D. in a Spectronic 20 set at 600 nm. A suspension of that turbidity had been previously determined to contain ca. 5×10^6 conidia/ml.

After the pods or seeds had been inoculated, the plates were swirled gently, enclosed in polyethylene bags and incubated at 25 C for indicated lengths of time.

The percentages of pods and seeds with visible colonies of A. flavus were noted after seven days as detailed in part I. The percentage of seeds invaded within inoculated pods and the shells penetrated were determined after 14 days of incubation generally as has been detailed in part I. Small pieces of scotch tape were used to isolate the fungus on the inner surface of the shell. Pods were incubated for 21 days after inoculation and then shelled to determine the colonization of seeds within inoculated pods.

Aflatoxin levels were estimated by the Tropical Products Institute method (33). Pods that had been incubated for 21 days following inoculation were dried at 50±2 C for 24 hours and shelled. The seeds (from two 20 g pod samples) were weighed and crushed in an analytical mill crusher. Twenty grams of the ground mixture were placed in extraction thimbles and extracted with hexane in Soxhlet apparatus for 4 hours. The remaining fat-free material was dried in a forced air oven at 105 C for 30 minutes and 10 g was placed in a 250 ml flask along with 100 ml chloroform and the flask was agitated for 30 minutes. The liquid was decanted and filtered. Ten μl of the filtrate was spotted on adsorbosil thin layer plates along with a 10 μl standard of aflatoxin B_1 (500 ppb). The plates were developed in diethylether to remove impurities (53), then allowed to air dry and finally developed in a chloroform: methanol (95:5) mixture.

To test for the presence of antagonistic microflora in the shells of certain peanut lines, the cured pods were hand-shelled. The shells (10 g) were blended in 100 ml sterile distilled water in a Waring Blender at low speed for 2-3 minutes. The resulting suspension was added to 100 ml rose bengal streptomycin agar (RBSA) media at 45 C then swirled and plated in 20 X 100 mm plastic petri dishes. After the agar had hardened, 5-6 ml of a suspension of *A. flavus* conidia in RBSA was added and the plate was swirled. The *A. flavus*-RBSA suspension had been prepared by pouring 5 ml of an aqueous suspension of 5 X 10^6 conidia/ml into 100 ml RBSA media maintained at 45 C. The antagonism of shell microflora on *A. flavus* was also studied by placing 2-3 mm blocks removed from the plates of RBSA-shell suspension prepared as above, on the plates of the *A. flavus*-RBSA suspension also prepared as above. The plates were incubated at 25 C for seven days and then the kind and the intensity of the fungal growth was evaluated.

RESULTS

The tolerance of seeds and pods of different peanut breeding lines to Aspergillus flavus. The percentage of colonization of seeds of the different breeding lines ranged from 13 to 64 in 1974 and 10 to 47 in 1975 (Table 9). The lines that were the most tolerant in 1974 were not the most tolerant in 1975. Breeding line No. 19, a line reported to be tolerant by LaPrade et al. (40) had the lowest percentage colonization in 1974 tests but did not have significantly lower colonization than the most susceptible lines in 1975. Breeding lines No. 13 and No. 17 appeared susceptible in 1974 but seemed tolerant in 1975.

The percentage of pods with A. flavus colonies varied among the different breeding lines tested (Table 10). As in the test with the hand-shelled seeds, the pods of some lines that were highly tolerant in 1974 were susceptible in 1975. The tolerance of pods was not correlated with the tolerance of seeds. Although tolerance was expressed here on the basis of the percentage of the pods with at least one sporulating colony of A. flavus, there were differences in the extent of colonies of the individual pods. On some pods almost the entire surface was colonized while on others single colonies were noted and on still others many scattered patches of colonies of different sizes were observed.

In both tests pods of each line were rehydrated and incubated with no inoculation. Several different fungi were noted growing from the pods in each years' test. In 1974, A. flavus occurred naturally on the pods of 80% of BRL No. 18 and No. 19, 75% of BRL No. 17, and 20% of BRL No. 21 and No. 20. In 1975, none of the uninoculated pods had visible colonies of A. flavus.

Influence of curing and sodium hypochlorite treatment. The presence of fungi in the shell may have interfered with the analysis of tolerance to A. flavus. When the field cured pods of a few lines were treated with sodium hypochlorite (NaOCl) to alter the surface microflora of the pod, and inoculated with A. flavus the percentage colonization of the pod increased significantly over that of the non-treated pods. A split plot analysis of the data indicated significant interaction between parts colonized and NaOCl treatment when the breeding lines (No. 1, 2, 7 and 16) with zero and 100% colonization were deleted from the analysis (Table 11). When the data for individual breeding lines were compared, pods of five out of 12 BRL tested had significantly higher percentage colonization of the pod.

In an attempt to reduce the pod contamination during field curing, the pods were dried rapidly in an oven immediately after harvesting and were inoculated with A. flavus. The oven dried pods of all the lines tested were completely colonized by A. flavus and were almost uniformly covered on the entire surface of the pods with A. flavus. The pods of the same lines when field cured were not uniformly colonized following A. flavus inoculation.

In general the per cent of invasion of seeds within inoculated pods of the different breeding lines was lower than the respective colonization of pods (Table 12). Although all of the inoculated oven dried pods were colonized, the invasion of the seeds within the pods of the different lines varied from 5-70%. The per cent invasion of seeds did not differ between drying periods of 12 hours and 48 hours, except in BRL no. 13.

The NaOCl treatment of the pods increased seed invasion in two peanut lines (No. 1 and 2) out of the 12 tested. However, a split plot analysis of the data indicated no significant difference between the percentage invasion of seeds from pods of those treated and those not treated with NaOCl (Table 11).

Influence of delayed inoculation at high pod moisture on percentage colonization of pods. Since the colonization of pods varied between inoculations, especially when the inoculation was delayed, the pods of a tolerant line were adjusted to 25% moisture and held at 25 C for 10 hours before inoculation. The percentage of pods colonized and seeds invaded decreased significantly if the pods were not inoculated until 10 hours following rehydration (Table 13).

Relation of colonization of pods and seeds to aflatoxin production in seeds. Two peanut lines that are tolerant to colonization of pods by *A. flavus* (BRL No. 19 and 21) and two that are susceptible (BRL No. 14 and 16) were either treated with NaOCl or non-treated with NaOCl and tested for the production of aflatoxin in the seeds following inoculation with *A. flavus*. In general, the lower percentages of colonization of pods and seeds by *A. flavus* in different peanut lines were associated with lower amounts of aflatoxin in the seeds (Table 14). However, BRL No. 14 had slighlty lower amounts of aflatoxin than that of BRL No. 21 when pods were not treated with NaOCl. The significant increases in the colonization of pods due to treatment of pods with NaOCl prior to inoculation were not associated with a significant increase in the production of aflatoxin except in BRL No. 14. In BRL No. 14, however, the significant increase in the quantity of aflatoxin was not associated with the significant changes in percentage colonization of seeds.

The fungi isolated from pods that were treated with NaOCl did not differ from those that are not treated except for *Aspergillus* sp. which was isolated only from the latter (Table 15). *Aspergillus* sp. and *Rhizopus* sp. were isolated only from the pods and not from the seeds within. Futhermore, *Trichoderma viridae* Person ex. Friez was isolated from seeds from pods that were non-treated but not from those treated with NaOCl.

Role of mycoflora of the shell on the tolerance of pods. The triturated shells of four peanut breeding lines with different levels of tolerance to colonization of pods by *A. flavus* were plated with *A. flavus* and the dominant fungi developing from the plates were noted. When *A. flavus* conidia were spread as a lawn over a shell-RBSA plate, the growth of *A. flavus* was suppressed to various degrees by the growth of fungi from pieces of the shells. The partial inhibition of *A. flavus* by other fungi was not necessarily associated with the pods of those breeding lines (No. 2 and 19) that were tolerant to colonization by *A. flavus*. In general, *Penicillium* sp., *A. niger*, and *Rhizopus* sp., were dominant in both the tests. When shell-RBSA blocks were placed on an *A. flavus*-RBSA plate and incubated for a week, fungi grew around many blocks; the clear zones around blocks, however, were formed only around two blocks of BRL No. 14. No fungal growth was observed on those blocks.

Table 9: The percentage colonization of seeds of 17 different peanut breeding lines grown in two different years, following inoculation with *Aspergillus flavus*.

BRL[x] No.	Percentage seed colonization 1974	1975
17	35 bcdef[y]	10 d
13	30 cdef	14 cd
14	24 def	19 bcd
7	41abcd	21 bcd
15	42abcd	22 bcd *[z]
2	54ab	23 bcd *
6	49abc	26abc
19	13 f	25abc
22	44abcd	26abc
16	19 ef	26abc
1	64a	29abc *
24	43abcd	30abc **
21	45abcd	31abc
18	34 bcde	33abc
9	27 def	37ab
20	30 cdef	40ab
26	64a	47a

[x] Identification of breeding line (BRL) included in appendix.

[y] Values (average of three 15 g seed samples) within columns followed by the different letters are significantly different ($\underline{P}$=0.05, by Duncan's New Multiple Range Test).

[z] Analysis of variance between years. *Significant at .05 level **Significant at .01 level.

Table 10: The percentage colonization of pods of 17 different peanut breeding lines grown in two different years, following inoculation with *Aspergillus flavus*.

BRL	Percentage pod colonization	
No.	1974	1975
1	3 e[x]	0 d
2	0 e	0 d
18	100a	0 d *[y]
22	23 c	2 d **
21	23 c	2 d **
24	16 e	2 d
19	88a	2 d **
9	3 e	2 d
6	0 e	3 d
26	2 e	3 d
20	4 e	7 d
13	0 e	34 c **
14	11 de	40 b
15	23 cd	56 b *
7	0 e	100a **
16	88 b	100a
17	100a	100a

[x] Values (average of three 15 g pod samples) within columns followed by the different letters are significantly different (P=0.05, by Duncan's New Multiple Range Test). Data was transformed to arcsin values for analysis.

[y] One way analysis of variance between years. *Significance at .05 level **Significant at .01 level.

Table 11: The percentage colonization of pods, and the percentage of invasion of seeds, of intact pods of 12 different peanut breeding lines treated or non-treated with sodium hypochlorite, and inoculated with *Aspergillus flavus*.

BRL No.	Percentage pod colonization		Percentage seed invasion	
	Non-treated[x]	Treated	Non-treated	Treated
75-1	0 d[y]	5 cd	0 e	12 c **
75-2	0 d	19 c *[z]	2 de	12 bc *
75-21	2 d	14 cd*	6 cde	13 c
75-19	2 d	7 cd	9 bcde	0 d**
75-9	4 d	7 cd	3 de	0 d
75-6	3 d	5 d	0 e	0 d
75-26	3 d	7 cd	13 bcd	3 d
75-13	34 c	64 b *	9 bcde	19 bc
75-14	40 bc	92a *	8 cde	27 b
75-15	56 b	79 b *	21abc	23 bc
75-16	100 a	100a	29ab	51a
75-7	100 a	100a	40a	53a

[x] Non-treated = pods that are not treated with NaOCl; Treated = pods that are treated with NaOCl.

[y] Values (average of three 20 g pod samples) within columns followed by the different letters are significantly different ($P=0.05$, by Duncan's New Multiple Range Test). Data was transformed to arcsin values for analysis.

[z] Analysis of variance within each breeding line between treated and non-treated for pods and seeds. *Significance at .05 level. **Significance at .01 level.

Table 12: The percentage of invasion of seeds of intact pods of seven peanut breeding lines dried for 12 or 48 hours, inoculated with *Aspergillus flavus*, and incubated for 14 days.[x]

BRL No.	Percentage seed invasion of pods dried	
	12 hours	48 hours
75-19	9 e[y]	5 d
75-15	12 de	16 cd
75-14	22 cd	37 bc
75-13	34 bc	53ab *[z]
75-7	59a	54ab
75-16	48ab	71a
75-18	51a	

[x] Pods of all breeding lines in both the drying treatments were 100% colonized following inoculation.

[y] Values (average of three 20 g pod samples) within columns followed by the different letters are significantly different (P=0.05, by Duncan's New Multiple Range Test). Data was transformed to arcsin values for analysis.

[z] Analysis of variance between drying periods. *Significant at .05 level.

Table 13: The percentage colonization of pods, and the percentage of invasion of shells and seeds of intact pods of peanut breeding line 74-7 adjusted to 25% moisture, incubated for zero or 10 hours and inoculated with *Aspergillus flavus*.

Parts colonized or invaded	Percentage colonization or invasion at preinoculation incubation period	
	0 hours	10 hours
Pod	65**[x]	10
Shell	25*	10
Seed	30*	12

[x] Analysis of variance between incubation periods. *Significant at .05 level. **Significant at .01 level.

Table 14: The amount of aflatoxin B_1 in seeds, and the percentage of colonization of pods and seeds of intact pods of four peanut breeding lines treated or non-treated with sodium hypochlorite, inoculated with _Aspergillus flavus_.[w]

BRL No.	Aflatoxin ppb. (X10)		Percentage colonization			
			Pod		Seed	
	T[x]	NT	T	NT	T	NT
75-19	26 b[y]	12 c	8 d	4 c	3 c	4 b
75-21	83 b	65 b	17 c	9 c*	14 bc	7 b
75-14	132a	42 bc*[z]	86 b	43 b *	19 b	10 b
75-16	137a	133a	100a	85a	37a	26a

[w] In both treated and non-treated checks neither _A. flavus_ nor aflatoxin was found.

[x] T = pods treated with NaOCl, NT = pods not treated with NaOCl.

[y] Values (average of 3 replications of two 20 g pod samples) within columns followed by the different letters are significant (P=0.05, by Duncan's New Multiple Range Test). Data on percentage of pod and seed colonization were transformed to arcsin values for analysis.

[z] Analysis of variance between treated and non-treated within the breeding line. *Significant at .05 level.

Table 15: The mycoflora of pods and seeds of intact pods of two peanut breeding lines treated or non-treated with sodium hypochlorite and incubated for 14 days.[x]

Fungi	Mycoflora of (BRL No.)							
	Pod				Seed			
	T[y]		NT		T		NT	
	14	16	14	16	14	16	14	16
Macrophomina phaseolina	+	+	+	+	+	–	+	–
Aspergillus niger	+	+	+	+	+	+	+	+
Aspergillus sp.	–	–	+	+	–	–	–	–
Rhizopus sp.	–	+	+	+	–	–	–	–
Penicillium sp.	+	+	+	+	+	+	+	+
Fusarium sp.	+	+	+	–	–	–	+	–
Sclerotium sp.	–	+	–	+	–	–	–	+
Trichoderma viridae	+	–	+	+	–	–	+	–

[x] Fungi isolated from the surface of the pod or seed (+ = isolated, – = not isolated)

[y] T = pods treated with NaOCl, NT = pods non-treated with NaOCl.

Table 16: The relative intensity of fungal colonies on the plates of RBSA media following plating of shells from pods of four different breeding lines with *Aspergillus flavus* by two different methods and incubation for seven days.

BRL No.	Af[x]	An	Rs	Ps	As	Fs	Mp	Tv
a. Shell - RBSA plate check								
75-2		+	+++	+		+	+	+
75-14		++	+	++	+	+	+	+
75-16		+	+	+++	+	+	+	
75-19				+++		+	+	
b. Shell - RBSA with A. flavus - RBSA								
75-2			+++					
75-14		++		+				
75-16	++	++		+++				
75-19	++		++	++				
c. Blocks from shell - RBSA plate placed on A. flavus - RBSA plate.								
75-2		2+[y]	2++					
75-14		4+		2+				
75-16				6+++				
75-19				8+++				

[x] Af=*A. flavus*, An=*A. niger*, Rs=*Rhizopus sp.*, Ps=*Penicillium sp.*, As=*Aspergillus sp.*, Fs=*Fusarium sp.*, Mp=*Machrophomina phaseolina*, Tv=*Trichoderma viridae*.

[y] Number of blocks out of 10 with fungus growth.

DISCUSSION

The percentage of seeds of different peanut breeding lines that developed visible colonies of Aspergillus flavus varied from 13-64% following inoculation with conidia in 1974 and 10-47% in 1975. Some of the lines that had lower colonization (tolerant) in 1974 had higher colonization (susceptible) in 1975. The BRL No. 19 which was reported to be tolerant to colonization of seed (40) was indeed tolerant in the 1974 test but was not very tolerant in the 1975 test.

The percentage of colonization of pods of the breeding lines tested varied from 0-100%. Pods of some of the breeding lines that were heavily colonized in 1974 were not colonized in 1975. The tolerance of the pods varied even within the same year among the different lines tested. For example pods of BRL No. 74-7 that were not colonized by A. flavus in an initial test (Table 10) were heavily colonized (65%) when inoculated in a test two months later (Table 13). Although the tolerance of pods of different peanut breeding lines to A. flavus was inconsistant between years, in each year the majority of the lines tested appeared tolerant (less than 10% colonization). The tolerance of pods of different peanut lines was not associated with the tolerance of the hand-shelled seeds of the same peanut lines.

Although all pods (100% colonization) of two lines in 1974 and three lines in 1975 had at least one colony of A. flavus, most of the individual pods were not uniformly colonized. Often the colonies of

A. flavus were restricted to a few patches of various sizes. Usually pods of the uninoculated checks were entirely covered by the colonies of fungi other than *A. flavus.* The colonies of certain species of fungi often occurred in patches of various sizes.

Mature pods are known to be devoid of cuticular layers which might provide protection against penetration by *A. flavus*. The epidermal layer is shed during the maturation process that occurs in the soil before harvesting. Thus the naturally occurring fungi on or in the shell could be the reason some pods appeared tolerant to *A. flavus*. When microflora on the outersurface of pods were altered by treatment with NaOCl, the percentage of pods with colonies following inoculation with *A. flavus* increased as did the number of colonies on the individual pods. As was expected, however, the growth of *A. flavus* was still not uniform over the entire surface of the pods since a surface treatment with NaOCl could not affect the microflora in the shell.

When the pods were immediately oven dried after being dug, all the lines were found to be highly susceptible. In addition almost the entire surface of many pods were uniformly covered with sporulating colonies of *A. flavus*. This suggested that field curing may be necessary for the expression of tolerance of peanut pods to *A. flavus*. The oven drying process did affect the mycoflora of the shell as uninoculated pods had only a few scattered colonies of mainly *Fusarium* sp. The reduction in mycoflora in the shell may be mainly due to drying of the pods immediately after harvest as most fungal contamination of the pod has been reported to occur during the process of curing in the field (45). When the microflora in the shell were given a chance to develope before *A. flavus* conidia were added, the tolerance of the pod was increased.

The dominant fungi isolated from the peanut shells included *Penicillium* sp., *A. niger* and *Rhizopus* sp. These fungi appeared to antagonize *A. flavus* either when a conidial suspension of the latter was spread as a lawn over the plate of triturated shells mixed with RBSA medium, or when agar blocks from a shell-RBSA plate were placed on an *A. flavus*-RBSA plate. In the latter test some agar blocks were associated with clear zones of inhibition and yet had no apparent fungal growth. Many other blocks had fungal growth and no apparent zone of inhibition of *A. flavus*.

Although there was an interaction between the fungi in the shell, especially the dominant ones mentioned above, and *A. flavus*, the specific nature of antagonism can not be determined with the available results. The antagonistic effect of certain fungi to *A. flavus* has been reported (10, 59). Wells *et al.* (59) have reported a reduction in pod colonization by *A. flavus* in the presence of *T. viridae* although the antagonism was nullified when *Penicillium funiculosum* was present. *Aspergillus niger* has been reported to reduce aflatoxin production by *A. flavus* (10) but its role in prevention of pod colonization has not been established. On the contrary, *A. flavus* has been reported to inhibit bacteria and fungi (24, 29).

Data presented in the above study do support the consideration that tolerance of pods of different peanut breeding lines is mainly due to mycoflora on or within the shell. However, the specific role that different fungi either alone or in combination play in reducing the colonization of pods or seeds by *A. flavus* needs further study.

In general, the percentage of seeds invaded within inoculated pods for all peanut lines were less than the respective percentage of pods with colonies. In addition, the percentage of shells that were penetrated was lower. But the entire inner surface of the shell was not sampled. So this may be an artifact. The lower number of seeds invaded may be due not only to mycoflora in the seed and/or to tolerance of the seeds (Table 9). Shells are also known to possess tanins which occurs either as pockets in the mechanical and conducting cells or as dark patches on the inner surface (52). Although tanins in the seed coat of certain peanut lines were reported to offer resistance to colonization by A. flavus (14), peanut seeds with tan colored testa (contain tanin) have been found to be as susceptible as those with white testa (38, 40). However, the role of tanin in tolerance of pod is not clear as the shell tanin differs chemically from that of sead coat tanin (52).

Tolerance of pods was associated with a lower concentration of aflatoxin in the seeds within inoculated pods. In addition, the amount of aflatoxin found in seeds was significantly higher in certain lines when the pods had been treated with NaOCl before inoculation. The latter treatment was associated with greater susceptibility of the pods. However, the relation of colonization of pods and seeds to the amount of aflatoxin produced in the seed is not very clear. This may be partly due to the variation in the amount of fungal (A. flavus) mycelium present in the seed as the percentage colonization of seeds do not indicate actual quantity of mycelia within the seed.

The tolerance of pods of peanut lines could be used commercially to reduce contamination of the seeds by A. flavus and/or aflatoxin. Since most colonization of the pods occurs after digging during the curing process (4, 45), the microflora addition in pods during the curing process may be so manipulated as to increase the influence of those that are antagonistic to *A. flavus*. The major limitation of such a biological control is that the pods should be intact for the maximum expression of tolerance. However, various types of tolerance should be an addition along with other precautionary measures advocated before (35, 43) to reduce contamination of the peanut seeds by *A. flavus*.

LITERATURE CITED

1. Asao, T., G. Buchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick, and G. N. Wogan. 1963. Aflatoxins B & G. J. Ameri. Chem. Soc. 85:1706-1707.

2. Ashworth, L. J., Jr. and B. C. Langley. 1964. The relationship of pod damage to kernel damage by molds in spanish peanuts. Plant Dis. Rep. 48:875-878.

3. Ashworth, L. F., Jr., H. W. Schroeder, and B. C. Langley. 1965. Aflatoxins: Environmental factors governing occurrence in spanish peanuts. Science 148:1228-9.

4. Austwick, P. K. C., and G. Ayerst. 1963. Toxic products in groundnuts: Groundnut microflora and toxicity. Chem. Ind. (London) 2:55-61.

5. Bampton, S. S. 1963. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts I. Tropi. Sci. 5:74-81.

6. Bell, D. K., and J. L. Crawford. 1967. A Botran amended medium for isolating *Aspergillus flavus* from peanuts and soil. Phytopathology 57:939-941.

7. Benedict, C. R., D. L. Ketring, R. E. Pettit, and J. W. Dieckert. 1973. Comparison of *Aspergillus flavus* tolerant and susceptible peanut lines. III Physiological investigation. J. Ameri. Peanut Res. & Education Assoc. 5:208.

8. Blount, W. P. 1961. Turkey 'X' disease. Turkeys (J. Brit. Turkey Federation) 9:55-58.

9. Bothast, R. J., and C. W. Hesseltine. 1975. Bright greenish-yellow fluorescence and aflatoxin in agricultural commodities. Appli. Microbiol. 30:337-38.

10. Burnett, C., and G. W. Rambo. 1972. Aflatoxin inhibition and detoxification by a culture filterate of *Aspergillus niger*. Phytopathology 62:749 (Abstr.).

11. Burreld, N. J., J. K. Grundey, and C. Harkness. 1964. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts-part V. Tropi. Sci. 6:74-90.

12. Butler, W. H. 1964. Accute toxicity of aflatoxin B_1 in rats. Brit. J. Cancer 18:756-762.

13. Carnaghan, R. B. A., R. D. Hartley, and J. O'Kelly. 1963. Toxicity and fluorescence properties of the aflatoxin. Nature 200:1101.

14. Carter, J. B. H. 1970. Studies on the growth of *Aspergillus flavus* on ground-nut kernels. Ph.D. Thesis Univ. of Reading (London, England) 170 p. Univ. Microfilms, Ann Arbor, Mich.

15. Davis, N. D., and U. L. Diener. 1968. Growth and aflatoxin production by *Aspergillus parasiticus* from various carbon sources. Appli. Microbiol. 16:158-159.

16. Davis, N. D., U. L. Diener, and K. E. Landers. 1964. Factors influencing the production of aflatoxin by *Aspergillus flavus* growing laboratory media. Proceedings of III National Peanut Research Conference. 144p.

17. Dickens, J. W., and H. E. Pattee. 1966. The effects of time, temperature, and moisture on aflatoxin production in peanut inoculated with toxic strain of *Aspergillus flavus*. Tropi. Sci. 8:11-22.

18. Dickens, J. W., and R. E. Welty. 1969. Detecting farmers' stock peanuts containing aflatoxin by examination for visible growth of *Aspergillus flavus*. Mycopathologia et Mycologia Applicata 37:65-69.

19. Dieckert, J. W., M. C. Dieckert, R. E. Pettit, C. R. Benedict, and D. L. Ketring. 1973. Comparison of *Aspergillus flavus* tolerant and susceptible lines. II Electronmicroscopy. J. Ameri. Peanut Res. & Education Assoc. 5:206 (Abstr.).

20. Diener, U. L., and N. D. Davis. 1965. Effect of temperature on aflatoxin production by isolates of *Aspergillus flavus* and *A. parasiticus*. Proceedings of IV National Peanut Research Conference 93.

21. Diener, U. L., and N. D. Davis. 1966. Aflatoxin production by isolates of *Aspergillus flavus*. Phytopathology 56:1390-93.

22. Diener, U. L., and N. D. Davis. 1967. Limiting temperature and relative humidity for growth and production of aflatoxin and free fatty acids by *Aspergillus flavus* in sterile peanuts. J. Ameri. Oil Chemists Soc. 44:259-263.

23. Diener, U. L., and N. D. Davis. 1968. Effect of environment on aflatoxin production in freshly dug peanuts. Tropi. Sci. 10:22-28.

24. Diener, U. L., and N. D. Davis. 1969. Aflatoxin formation by Aspergillus flavus, p. 13-54. In L. A. Goldblatt, Aflatoxin Scientific Background, Control, and Implications. Academic Press, New York and London.

25. Diener, U. L., and N. D. Davis. 1972. Atmospheric gasses and aflatoxin production in peanuts. Highlights of Agr. Res. 19:3.

26. Diener, U. L., N. D. Davis, W. D. Salmon, and C. O. Prickett. 1963. Toxin producing Aspergillus isolated from domestic peanuts. Science 142:1491-1492.

27. Doupnik, B., Jr. 1969. Aflatoxin produced on peanut varieties previously reported to inhibit production. Phytopathology 59:1554.

28. Jackson, C. R. 1965. Laboratory evaluation of fungicides for control of some fungi found on peanuts. Plant Dis. Rep. 49:928-935.

29. Jackson, C. R. 1965. Peanut kernel infection and growth in vitro by four fungi at various temperatures. Phytopathology 55:46-48.

30. Jackson, C. R. 1965. Growth of Aspergillus flavus and other fungi in windrowed peanuts in Georgia. Tropi. Sci. 7:27-34.

31. Jackson, C. R. 1967. Some effects of harvesting methods and drying conditions on development of aflatoxins in peanuts. Phytopathology 57:1270-71.

32. Jackson, C. R. 1968. A field study of fungal associations on peanut fruit. Ga. Agr. Expt. Sta. Res. Bull. 26 Athens.

33. Jones, B. D. 1972. Methods of aflatoxin analysis. Tropical Products Institute Report No. G 70.

34. Karon, M. L., and B. E. Hillery. 1949. Hygroscopic equilibrium of peanuts. J. Ameri. Oil Chemists Soc. 26:16-19.

35. Kensler, C. J., and D. J. Natoli. 1969. Processing to ensure wholesome products. p. 333-353. In L. A. Goldblatt, Aflatoxin Scientific Background, Control, and Implications. Academic Press, New York and London.

36. Kulkarni, L. G., Y. Sharief, and V. S. Sarma. 1967. "Asiriya mwitunde" groundnut gives good results at Hyderabad. Indian Farming 17:11-12.

37. Lancaster, M. C., F. P. Jenkins, and J. Philip. 1961. Toxicity associated with certain samples of groundnuts. Nature 192:1095-1096.

38. LaPrade, J. C. 1973. Physical and chemical properties of resistance exhibited by certain genotypes of *Arachis hypogaea* to invasion by aflatoxin producing *Aspergillus spp.* Ph.D. Thesis Univ. of Florida. pp. 77.

39. LaPrade, J. C., and J. A. Bartz. 1972. Mechanical resistance of selected genotypes of dried peanuts to colonization by strains of aflatoxin producing *Aspergillus spp*. Phytopathology 62:771 (Abstr.).

40. LaPrade, J. C., J. A. Bartz, A. J. Norden, and T. J. Demynk. 1973. Correlation of peanut seed coat surface wax accumulation with tolerance to colonization by *Aspergillus flavus*. J. Ameri. Peanut Res. & Edu. Assoc. 5:89-94.

41. Lee, L. S., L. Y. Yatsu, and L. A. Goldblatt. 1967. Aflatoxin contamination: Electron microscopic evidence of mold penetration. J. Ameri. Oil Chemists Soc. 44:331-332.

42. McDonald, D., and J. A. Brook. 1963. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts part III. Tropi. Sci. 5:208-214.

43. McDonald, D., and C. Harkness. 1963. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts part II. Tropi. Sci. 5:143-154.

44. McDonald, D., and C. Harkness. 1964. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts part IV. Tropi. Sci. 6:12-27.

45. McDonald, D., and C. Harkness. 1965. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts part VIII. Tropi. Sci. 7:122-137.

46. Mixon, A. C., and K. M. Rogers. 1973. Breeding peanuts for resistance to *Aspergillus flavus*. Agronomy J. 65:560-562.

47. Mixon, A. C., and K. M. Rogers. 1975. Factors affecting *Aspergillus flavus* Link. Colonization of resistant and susceptible genotypes of *Arachis hypogaea* L. Peanut Science. 2:18-22.

48. Pattee, H. W., and S. L. Sessoms. 1966. Relationship between *Aspergillus flavus* growth, fatty acid, aflatoxin content in peanuts. J. Ameri. Oil Chemists Soc. 44:61-63.

49. Pettit, R. E., R. A. Taber, H. W. Shroeder, and A. C. Harrison. 1971. Influence of fungicides and irrigation practice on aflatoxin in peanuts before digging. Appli. Microbiol. 22:629-34.

50. Sandars, T. H., N. D. Davis, and U. L. Diener. 1968. Effect of carbondioxide, temperature, and relative humidity on production of aflatoxin in peanuts. J. Ameri. Oil Chemists Soc. 45:683-685.

51. Sargeant, K., A. Sheridan, J. O'Kelly, and R. B. A. Carnaghan. 1961. Toxicity associated with certain samples of groundnuts. Nature 192:1096-1097.

52. Schenk, R. U. 1960. Source of the inner brown color of the peanut shell. Bot. Gaz. 121:191-192.

53. Shcroeder, H. W. 1968. Metabolite of Macrophomina phaseoli that can confuse thin layer chromatographic identification of aflatoxin B_2. Appli. Microbiol. 16:946-47.

54. Shotwell, O. L., C. W. Hesseltine, R. D. Stubblefield and W. G. Sorenson. 1966. Production of aflatoxin on rice. Appli. Microbiol. 14:425-428.

55. Smith, B. W. 1950. Arachis hypogaea. Aerial flower and subterranean fruit. Ameri. J. Botany 37:802-15.

56. Suryanarayana, R. K., and P. G. Tulpule. 1967. Varietal differences of groundnut in the production of aflatoxin. Nature 214:738-739.

57. Taber, R. A., R. E. Pettit, C. R. Benedict, J. W. Dieckert, and D. L. Ketring. 1973. Comparison of Aspergillus flavus tolerant and susceptible peanut lines. J. Ameri. Peanut Res. & Edu. Assoc. 5:206 (Abstr.).

58. Wells, T. R., W. A. Kreutzer. 1972. Aerial invasion of peanut flower tissue by Aspergillus flavus under gnotobiotic conditions. Phytopathology 62:797 (Abstr.).

59. Wells, T. R., W. A. Kreutzer, and D. L. Lindsey. 1972. Colonization of gnotobiotically grown peanuts by Aspergillus flavus and selected interacting fungi. Phytopathology 62:1238-41.

60. Wilson, B. J., T. C. Campbell, A. W. Hayes, and R. T. Hanlin. 1968. Investigations of reported aflatoxin production by fungi outside the Aspergillus flavus group. Appli. Microbiol. 16:819-821.

APPENDIX 1: Identification of the peanut breeding lines.

Laboratory BRL No.	Cross No./cultivar	University of Florida No.	
		1974	1975
1	519	102	102
2	520B	104	104
6	427B	111	111
7	420	112	112
9	487A	114	114
13	439	4001	4001
14	439	4005	4005
15	439	4009	4009
16	F439	4021	4021
17	Altika	4027	Altika
18	VF71-4021	4039	109
19	Val. 803	4045	4045
20	UF70-115	4055	115
21	Tifspan	504	504
22	Starr	502	502
24	Tamnut	509	509
26	UF73-515	515	515

BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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